Hydrophilic extractives from the needles of Scots pine and Norway spruce

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SUMMARY: The chemistry and techniques of identification of hydrophilic extractives (carbohydrates, mainly mono-, oligosaccharides and inositols, and phenolic compounds) in the needles of Scots pine *(Pinus sylvestris)* and Norway spruce *(Picea abies)* are presented. Many different types of phenols are found, including benzoic acids, acetophenones, phenylglycerols, cinnamic acids, dilignols, stilbenes and flavonoids, which mainly are present as glycosides with D-glucose, L-rhamnose, D-xylose or L-arabinose. Many compounds are common for the two species, but especially acetophenones and stilbenes are characteristic for spruce.

Such a detailed knowledge of the chemistry of needles is for instance of importance in connection with full tree utilization and parasite problems. The chemical composition of brown pine needles (ca. 25 % of the needles on the tree) and the changes of composition in pine-needle litter during a five-years period has been studied. Decomposition studies of this type are needed to elucidate problems concerning the accumulation and microbial activity of organic matter in the forest soil. Some of the flavonoids, for instance, have a significant growth-stimulating effect on typical decomposers of coniferous litter. Present promising studies on the possible use of phenolic extractives as biochemical markers in genetic studies show notable differences between pine clones.

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We have been interested in the hydrophilic extractives (water-soluble part of an ethanol or acetone extract) — low-molecular carbohydrates and phenols — of conifer needles for many years (1, 2). For a recent review of different classes of phenols in *Pinaceae* family needles in general, see (3). When this work was started at the Swedish Forest Products Research Laboratory on needles from *Pinus sylvestris,* we found that lipophilic extractives of conifer needles had been extensively studied, whereas the chemical characterization of hydrophilic carbohydrate and phenolic extractives was very incomplete.

Pine needles are, in particular, characterized by a complex mixture of phenolic glycosides of different sugars. Combinations of chromatographic fractionation methods with nuclear magnetic resonance (NMR), mass spectrometry (MS) and other identification methods have been invaluable means in the identification of these and other components. The pattern of the extractives from Norway spruce which we have started to study more intensively in recent years, differs quite considerably from that of Scots pine — for instance, the presence of a series of acetophenone and stilbene glucosides is characteristic.

More recent or current studies made in co-operation with other departments at the Faculties of Agriculture and of Forestry at this University on the chemical changes of pine needles when turning brown on the tree and as litter, on the effect of needle extractives on fungal growth, and on the possible use of phenolic extractives as biochemical markers in genetic studies will also be briefly presented.

Needles of Scots pine

Green needles

In earlier studies, when we used carbon column chromatography and gradient elution with aqueous ethanol as the main fractionation method, a series of low-molecular carbohydrates were identified from pine needles, including two phenolic glucosides (1, 2). Among these, some components, such as glucose, fructose, sucrose, pinitol *2*[1] and shikimic acid *3* (see *scheme 1*) were isolated in a yield of 1.5—2.5 % each of the dry weight. Arabinose, rhamnose, mannitol *1*, melibiose, raffinose, myo-inositol and sequoitol were isolated in the range of 0.1—1 %. Laminaribiose and cellobiose were found, for the first time, as free disaccharides, together with some other minor, unidentified oligosaccharides (1). At that time, acidic oligosaccharides were removed by ion-exchange resins, but in recent studies we have also isolated two acidic oligosaccharides, consisting of galacturonic acid and rhamnose (4).

Also isolated in the previous studies (2) were the *threo*-form of guaiacyl glycerol *4* (absolute configuration not determined) and the β-D-glucopyranosides of the latter, with the sugar linked in the α- and β-positions respectively in the glycerol side-chain. In a recent study (5) using chromatography on Sephadex LH-20, anion exchange and silicic acid chromatography, the γ-isomer of compound *4* (a mixture of *erythro*- and *threo*-isomers) was also isolated, as well as the β- and γ-β-D-glucopyranosides of *p*-hydroxyphenylglycerol *5*. It was shown that the β-compound was a mixture of *erythro*- and *threo*-isomers, but the γ-compound was isolated as a pure *threo*-isomer. Also compound *5* was present in free form, isolated as a pure *threo*-isomer. The amounts of individual compounds isolated in this group of extractives were 0.1 % or less. They are also present in other parts of the tree and may be of importance in connection with the biosynthesis of lignin.

Phenolic glycosides and other hydrophilic phenolic compounds may be enriched from the aqueous part of the acetone extract by extraction with 2-butanone. We found that fractionation on Sephadex LH-20 columns and elution with water and aqueous ethanol has a high capacity. It is very useful for crude fractionation of hydrophilic extractives from plant materials as well as of acidic and aromatic reaction products from thermal treatment of carbohydrates, for example (6). When a 2-butanone fraction of pine needles was fractionated in such a way, compounds were eluted in the following order (7):

[1] Italicised numbers designate compounds.

— mono- and oligosaccharides, sugar alcohols, cyclitols

— shikimic acid

— the glucosides of *4* and *5*

— dilignol glycosides

— 2,3-dihydroquercetin-3'-*O*-*β*-D-glucopyranoside *18*

— quercetin-3'-*O*-*β*-D-glucopyranoside

— 2,3-dihydroquercetin (taxifolin) *17*

— quercetin; and

— catechins.

Compounds *17, 18,* quercetin and its glucoside, and (+)-catechin *19* from the catechin mixture were identical with authentic compounds previously isolated from conifers (8). Some other flavonoids have also been found in *Pinus sylvestris* (see references in ref. 3). Glucoside *18* was isolated in over 2% of the dry weight. We did not make any systematic studies of the catechins (procyanidins), studied by Thompson et al. (9), both in *Pinus sylvestris* and *Picea abies.*

The complex mixture of dilignol glycosides was separated and obtained in a pure state after repeated chromatographic fractionation. *Fig. 1* (7) is an idealized schematic illustration of the chromatographic properties on which the purification procedure is based (silicic acid and Sephadex LH-20 chromatography)

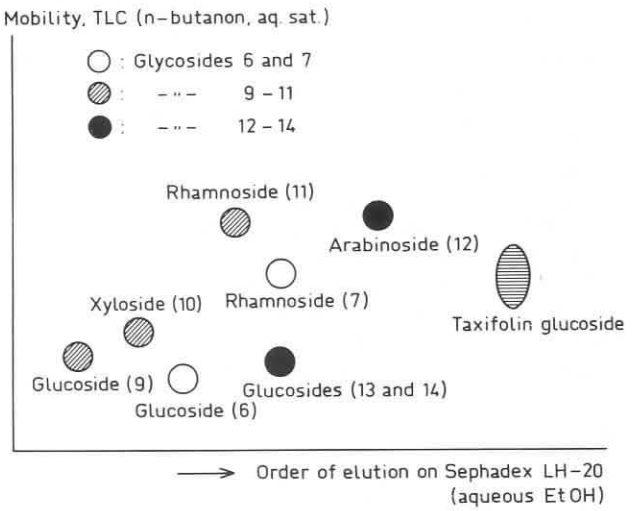
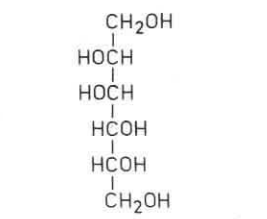


Fig. 1. Fractionation of glycosides from Scots pine needles.

Scheme 1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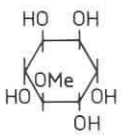
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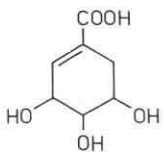
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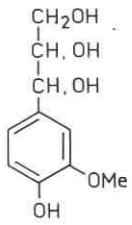
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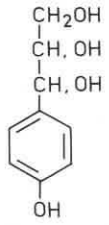
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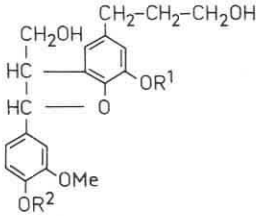
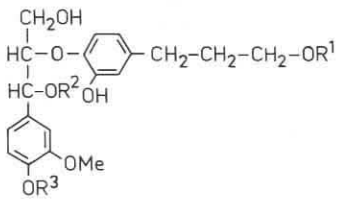
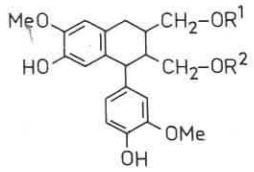
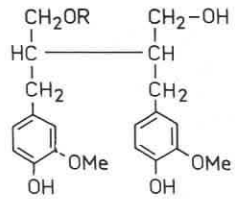
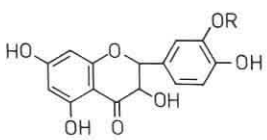
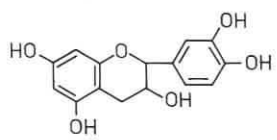

6 R^1 = H
R^2 = β-D-glucopyr.
7 R^1 = H
R^2 = α-L-rhamnopyr.
8 R^1 = Me
R^2 = β-D-glucopyr.


9 R^1 = R^3 = H R^2 = β-D-glucopyr.
10 R^1 = R^2 = H R^3 = β-D-xylopyr.
11 R^2 = R^3 = H R^1 = α-L-rhamnopyr.


12 R^1 = H R^2 = α-L-arabinofur.
13 R^2 = H R^1 = β-D-glucopyr.
14 R^1 = H R^2 = β-D-glucopyr.
15 R^1 = H R^2 = β-D-xylopyr.


16 R = β-D-glucopyr.


17 R = H
18 R = β-D-glucopyr.


19 R = H

of eight of the glycosides (and compound *18*) later identified (8).

It is interesting to note that a hexoside of a certain aglycone (such as the glucoside *9*) is eluted from the Sephadex LH-20 column before the corresponding pentoside (such as the xyloside *10*), and the latter before the 6-deoxy-hexoside (as the rhamnoside *11*). In a similar way, glucoside *6* is eluted before rhamnoside *7*, and glucosides *13* and *14* before arabinoside *12*.

In thin-layer chromatography (or column chromatography) on silicic acid, on the other hand, rhamnoside *11* has a much higher mobility than the glucoside *9*. The arabinoside *12*, being furanosidic, also exhibits a characteristical, high mobility compared with the pyranosidic xyloside *10*. The structures of nine dilignol glycosides (*6, 7, 9—14* and *16* given in scheme 1), including four types of aglycones, were identified (8). The aglycones corresponding to *6, 7* and *9—11* respectively were also isolated in free forms.

In addition to NMR and MS for the identification in general, methylation and studies of the products after subsequent hydrolysis were important for showing the position of the glycosidic linkage to the aglycone, and for proving whether the sugar part is pyranosidic or furanosidic.

The glycosides *9—11* represent a notable example of three different sugars linked to hydroxyl groups in the same aglycone, but in three different positions. We looked carefully for the other combinations without finding any indication of their presence. Compounds *9* and *11* had a *threo*-configuration in the glycerol chain while compound *10* was isolated as a *threo*-and *erythro*-mixture in the ratio 1:2. The fact that one of the aromatic rings is catecholic is a notable fact for this group of glycosides, as also for compounds *6* and *7*. The predominant dilignol glycosides were compounds *6, 7, 9* and *10,* isolated in 0.2—-0.4 % yield.

Other phenolic extractives from needles of *Pinus sylvestris* have also been isolated, namely caffeic, homoprotocatechuic and chlorogenic acids and also glucosides and/or the free form of *p*-hydroxybenzoic, vanillic, protocatechuic, *p*-coumaric and ferulic acids (see references in ref. 3).

Needle phenols as biochemical markers

So far monoterpenes have, almost exclusively, been used as biochemical markers, as for instance in chemosystematic and genetic studies. In view of recent improvements also in the gas-chromatographic (GC) determination of more high-molecular terpenes, the wider use of such markers would be of great interest. In our present studies we are investigating the possibility of developing a rather rapid technique of using phenolic constituents as biochemical markers (10) since there is a need for different types of biochemical markers as a complement to the isoenzyme technique in selecting plus trees, for seed control and so on. The use of phenols appears attractive also because there may be important relations between the composition of phenolic constituents and resistance to parasites.

In order to simplify the method we are studying the pattern of free phenols — those originally present together with those aglycones which are released from the glycosides by an enzymatic treatment. At present we are studying such mixtures by two-dimensional thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC). *Fig. 2* shows as an example HPLC-graphs of part of the phenols from the needles of two different clones collected at the same time from the same stand. Although we must remember that different compounds may differ in detector response, a marked difference between the two clones is obvious, the variations of taxifolin *17* — peak D — being most predominant. Peaks A and B represent *p*-hydroxybenzoic and vanillic acid respectively, and peaks C and E probably epicatechin and seco-isolariciresinol respectively.

Brown needles and litter

In other work we studied the differences in the composition of low-molecular carbohydrates, cyclitols and phenolic glycosides — and also of lipophilic extractives, polysaccharides, lignin and crude protein — in green and brown Scots pine needles from the same stand (11, 12). We recently finished a study where we studied the decomposition and organic chemical

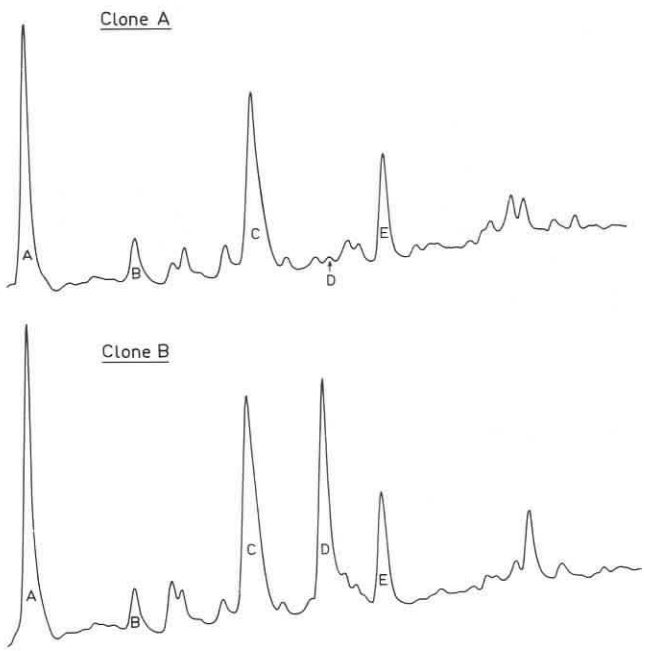


Fig. 2. HPLC separation of phenolic compounds from different clones of **Pinus sylvestris** *(μ-Bondapak C_{18}; linear gradient $H_2O \rightarrow 50$ % aq. methanol with a constant conc. of 2.5 % acetic acid; flow rate: 1.6 ml/min; UV-detector: 254 nm).*

changes for a period of five years (13). Since a high proportion of the needles on the tree are brown (around 25 %), it is of interest — for example, in connection with whole-tree utilization — to obtain knowledge about the chemical composition of that part of the foliage. In particular, a better understanding of the chemistry of dead needles and leaves and their further decomposition in the litter stage is important in the study of problems concerning the accumulation, decomposition, and microbial activity of organic matter in soil. Some examples from these studies on hydrophilic extractives are given in *table 1* (11).

Table 1. Some hydrophilic extractives from pine needles (% on dry weight basis; t = traces)

Compound	A	B	C	D
Glucose	2.3	0.4	0.3	0.2
Fructose	1.6	0.5	t	—
Sucrose	0.7	0.2	t	—
Mannitol *1*	1.8	0.1	0.2	0.1
Pinitol *2*	2.2	0.4	0.1	0.1
Myo-inositol	0.3	t	t	t
Glycoside *7*	0.3	0.4	t	t
Glycoside *18*	0.3	0.3	t	t

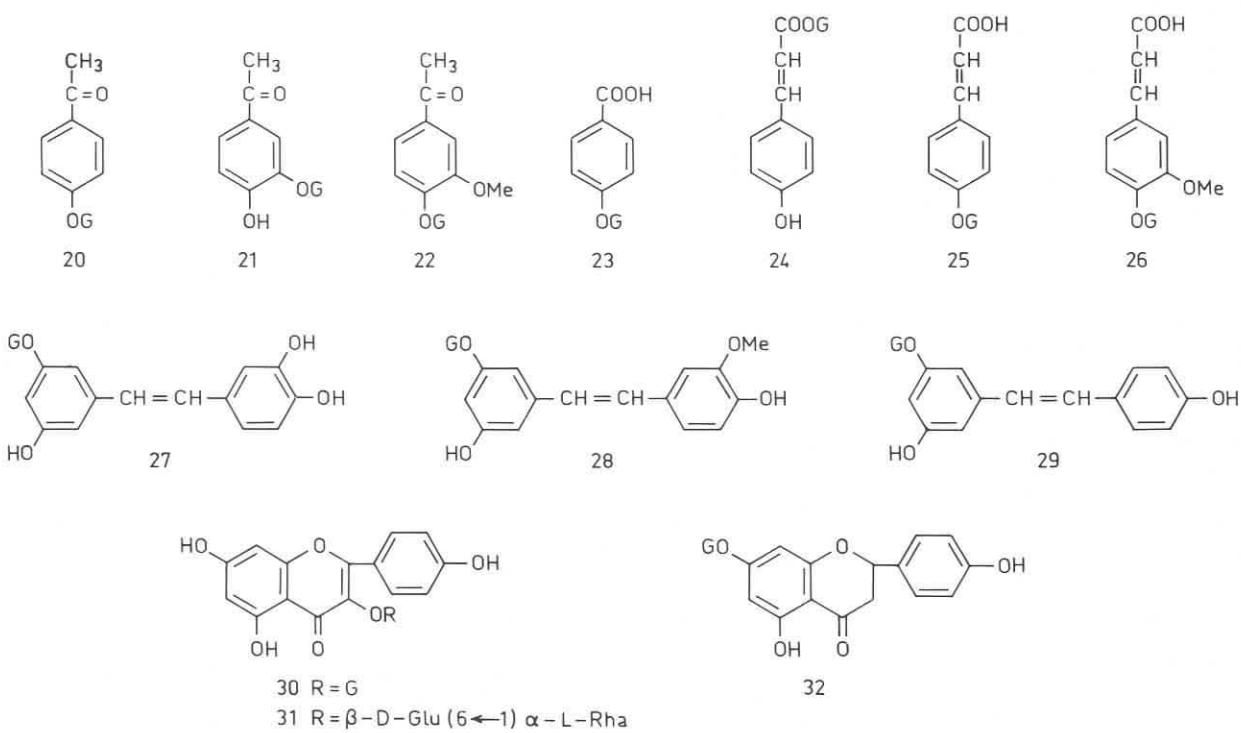
A = green needles and B = brown needles (just before the peak of needle litter-fall) — from the same tree. C and D are B-needles stored on the forest floor for 105 and 165 days respectively, resulting in drop in dry weight of 10.4 and 17.8 % respectively.

There is a drastic drop in the amount of sugars and cyclitols passing from green to brown needles. The small amounts of glucose and mannitol which we found in the litter even after a longer time probably reflect microbial degradation and synthesis. The amount of more condensed catechins and other non-identified phenolic compounds, which are enriched in an ethyl acetate fraction, seems to increase steadily. From the corresponding detailed study of the lipophilic extractives it should be mentioned that there is a great drop in the concentration of steryl esters and triglycerides from green to brown needles on the tree, followed by a continued decrease in the litter (12, 13). Pinifolic acid, which we identified as one of the major terpenoid constituents in pine needles (14), as well as some isoprenoid alcohols and sterols were among the most stable constituents in the litter study.

Needles of Norway spruce

More recently, we started studies similar to those discussed above on needles from Norway spruce *(Picea abies)*. From that source are previously reported (see references in ref. 3) the acetophenone derivatives picein *20* and pungenin *21* (see *scheme 2*), free protocatechuic, vanillic, gallic, syringic, *p*-coumaric, caffeic, ferulic, and chlorogenic acids as well as the glucoside of *p*-hydroxybenzoic acid *23* and *O*-coumaryl-glucose. Also reported (see ref. 3) are the stilbenoids

Scheme 2 (G = β-D-glucopyranoside)



27 and *28* and the flavonoids kaempferol (*30* and its 7-isomer) and quercetin glucosides as well as (+)-catechin, epicatechin and gallocatechin.

In the present studies we have not yet made any systematic investigation of low-molecular carbohydrates, including cyclitols, but glucose, fructose, sucrose, pinitol *2* and shikimic acid *3* are major components. The phenolic constituents (except dilignol compounds) so far isolated and identified (see scheme 2) — besides the compounds *19, 20, 21, 23, 27, 28* and *30* previously reported (3) from *Picea abies* — are the β-D-glucopyranosides, linked in the γ-position in the glycerol side-chain, of *4* and *5,* the β-D-glucopyranosides *22, 24, 25, 26* and *29* as well as the flavonoids *31* and *32* (15). We also isolated a series of dilignol glycosides (compare scheme 2) — but in lower yield than from *Pinus sylvestris* (total amount about 0.3 %). These include compounds *6, 7, 9, 10* and *16,* previously isolated from the latter source as well as the new glycosides *8* and *15* (16). Notably, a *threo-erythro* mixture of *10,* but only the *threo* isomer of *9* were isolated, which agrees with the results from the investigation of *Pinus sylvestris* (8). It is notable that the isolariciresinol aglycone in compound *15* has the (—)-configuration but compounds *12—14* have the opposite configuration.

Effect of needle extractives on fungal growth

In connection with the studies on green and brown needles from Scots pine, tests were made on the effect of extracts from these on the growth of several fungus species isolated from the same site as the needles (12). The acetone-soluble extractives from both types of needles were divided into petroleum ether, ethyl acetate and 2-butanone extracts, and an aqueous residue. There was an inhibition of growth, compared with the control, of most of the fungal strains particularly for the ethyl acetate extract, where many types of phenolic compounds are enriched. The inhibitory effect was, for instance, very strong on the common fungi *Cladosporium herbarum, Trichoderma polysporum* and *viride.* The effect was generally very similar, with some few exceptions, for extracts from green or brown needles. By testing subfractions (from a Sephadex LH-20 column) of the ethyl acetate extract from brown needles, indications were obtained that unidentified, probably condensed phenolic components were more inhibiting than identified phenolic glycosides and low-molecular catechins.

On the other hand, we found a stimulating effect of hydrophilic pine needle extractives on the growth of litter-decomposing basidiomycetes (17). Thus, taxifolin glucoside *18,* which sometimes constitutes 1—2 % of the dry weight of the needles, significantly enhanced the growth rate of *Marasmius androsaceus* at a concentration of 5 ppm. We found that many other flavonoids had a similar effect. *Collybia peronata* and *Micromphale perforans,* which are also known as needle-decomposing fungi, were also stimulated by taxifolin glucoside.

The pine parasite fungus *Lophodermium pinastri* was strongly stimulated by the aqueous extract from green needles of Scots pine (11, 18). Such extracts from green needles of Norway spruce also have a strong stimulating effect on the growth of the typical spruce-needle decomposing fungus *Marasmius perforans* and the spruce parasite *Scleroderris lagerbergii* (11, 17).

Acknowledgements

The good and stimulating co-operation with all colleagues and coworkers, cited in this paper, is gratefully acknowledged.

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